

# MEMD, a New Cell Adhesion Molecule in Metastasizing Human Melanoma Cell Lines, Is Identical to ALCAM (Activated Leukocyte Cell Adhesion Molecule)

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From a differential mRNA display comparing a non- and a highly metastasizing human melanoma cell line, we isolated and characterized *memD*. *memD* is preferentially expressed in the highly metastasizing melanoma cell lines of a larger panel. The encoded protein, MEMD, is identical to activated leukocyte cell adhesion molecule (ALCAM), a recently identified ligand of CD6. ALCAM is involved in homophylic (ALCAM-ALCAM) and heterophylic (ALCAM-CD6) cell adhesion interactions. We have studied MEMD/ALCAM cell-cell interactions between human melanoma cells. The expression of this cell adhesion molecule not only correlates with enhanced metastatic properties and with aggregational behavior of human melanoma cells as tested by FACS analysis, but transfection experiments also make clear that MEMD/ALCAM expression is essential for cell-cell interaction of the investigated human melanoma cells. As the melanoma cell lines analyzed are all CD6 negative, these results strongly suggest that MEMD/ALCAM is an adhesion molecule mediating homophylic clustering of melanoma cells. MEMD/ALCAM expression is not restricted to subsets of leukocytes and melanoma cells, it can also be found in healthy organs and in several other malignant tumor cell lines. Besides, MEMD/ALCAM is also expressed in cultured endothelial cells, pericytes and melanocytes, in xenografts derived from the radial and vertical growth phase and in 4 of 13 melanoma metastasis lesions. The potential role is discussed of MEMD/ALCAM mediated cell-cell interactions in migration of mobile cells (ie, activated leukocytes, metastasizing tumor cells) through tissues. (*Am J Pathol* 1998, 152:805-813)

ity, and invasion during tumor progression and metastasis.<sup>1</sup> In human cutaneous melanoma changes in the expression profiles of several adhesion molecules were observed that directly or inversely correlated with metastatic potential.<sup>2</sup> For example, the adhesion molecule E-cadherin is expressed on melanocytes and mediates adhesion to keratinocytes. Loss of this adhesion molecule enhances the motility of melanocytes and is, therefore, a marker of tumor progression.<sup>3</sup> However, the expression of melanoma cell adhesion molecule (Mel-CAM/MUC18) and intercellular adhesion molecule (ICAM-1) increased with malignant transformation in advanced primary and metastatic melanomas.<sup>4-6</sup> Likewise, the increased expression of the  $\alpha_2\beta_1$ ,  $\alpha_3\beta_1$ , and  $\alpha_4\beta_1$  integrins<sup>7-9</sup> is associated with invasion and metastasis of melanoma.

We have recently reported on the isolation of several cDNA clones that were differentially expressed in a panel of human melanoma cell lines with different metastasizing potential.<sup>10</sup> One clone, *memD* (this study), is preferentially expressed in the highly metastasizing human melanoma cell lines BLM and MV3 and is identical to activated leukocyte cell adhesion molecule (ALCAM).<sup>11</sup>

ALCAM, expressed on activated leukocytes T cells, B cells, and monocytes, was recently characterized as a new member of the immunoglobulin superfamily and identified as a CD6 ligand.<sup>11</sup> ALCAM is a type I membrane protein, it contains a NH<sub>2</sub>-terminal hydrophobic signal peptide, and is followed by an extracellular domain that is composed of five Ig-like domains: two amino-terminal V-type domains followed by three C-type domains, a hydrophobic transmembrane domain, and a short cytoplasmic tail. ALCAM has substantial homology with the chicken neural adhesion molecule BEN/SC1/DM-GRASP<sup>12-14</sup> and similarity with the human melanoma cell adhesion molecule Mel-CAM/MUC18.<sup>15</sup> BEN/SC1/DM-GRASP is involved in neurite extension and was also reported to be expressed on activated chicken leukocytes.<sup>16</sup> Several homologs of BEN/SC1/DM-GRASP/ALCAM have been identified in other species. Various

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types of cell adhesion interactions of BEN/SC1/DM-GRASP and of ALCAM were demonstrated.<sup>11,13,17</sup>

In the present study, we describe the characterization of *memD* as a new adhesion molecule preferentially expressed in highly metastasizing human melanoma cell lines and show that expression of *memD*/MEMD is essential for cell-cell interactions of the investigated human melanoma cells.

## Materials and Methods

### Biological Materials

All cell lines were grown and cultured as described before.<sup>18,19</sup> Within the panel of human melanoma cell lines, BLM and MV3 represent highly metastasizing cell lines with a metastasis frequency over 50%, 3 weeks after subcutaneous inoculation into nude mice. The cell lines M14, MV1, and Mel57 represent the slower metastatic phenotype with a metastasis frequency over 50% after three months. 530 and 1F6 are the nonmetastasizing cell lines with less than 10% metastasis frequency<sup>20-22</sup> (H. Westphal, unpublished results).

Excision and processing of the human tissues was performed as described previously.<sup>23</sup>

The monoclonal anti-ALCAM antibodies J3-119 and J4-81 are from the 5th International Workshop on Human Leukocyte Differentiation Antigens (November 1993, Boston, MA).<sup>24</sup>

### RNA Isolation and Northern Blot Analysis

Total RNA isolation and Northern blot analysis were all performed as described before.<sup>25</sup> Briefly, glyoxylated total RNA samples (10 µg) were loaded in each lane, transferred to Hybond N-plus filters, and hybridized to radiolabeled *memD* cDNA. The molecular weight marker was *Hind*III-digested λDNA. As a control for the amount of RNA loaded in each lane, an 18S rRNA hybridization is shown.

Other procedures were essentially the same as described before.<sup>25</sup>

### DNA Isolation and Southern Blot Analysis

Chromosomal DNA isolation and Southern blot analysis were all performed as described before.<sup>25</sup> Briefly, DNA was digested with *Eco*RI, and 10 µg was size fractionated on 1% agarose gels, transferred to Hybond N-plus filters, and hybridized to radiolabeled *memD* cDNA. The molecular weight marker was *Hind*III-digested λDNA. To confirm that equal amounts of DNA were loaded in each lane, the blot was hybridized afterwards to a chromosome 18-specific centromeric probe.<sup>26</sup> Abnormalities involving chromosome 18 have not been described in relation to melanoma.

### Immunofluorescence Assay

For the immunofluorescence studies, cells were grown on 12-well multitest slides (ICN, Costa Mesa, CA) and subsequently processed following the methanol-acetone fixation protocol.<sup>27</sup> Slides were incubated for 120 minutes with the monoclonal antibody J3-119 diluted 1:500 in phosphate-buffered saline (PBS).<sup>24</sup> Thereafter, the cells were washed with PBS, followed by a 60 minutes incubation with fluorescein-conjugated rabbit anti-mouse Ig antibody (diluted 1:50 in PBS, Dakopatts, Glostrup, Denmark). All incubations were performed at room temperature. After extensive washing with PBS (3 × 15 minutes), cells were mounted with PBS/glycerol (1:1, v/v).

### Measurement of Melanoma Cell Aggregation

#### Reagents

5,6-Sulfofluorescein diacetate (SFDA) and hydroethidine (HE) were purchased from Molecular Probes (Junction City, OR) and Polysciences (Warrington, PA), respectively. Stock solutions of SFDA (5 mg/ml in dimethyl sulfoxide) and HE (80 mg/ml in *N,N*-dimethylacetamide) were kept in the dark at 4°C. The working solution of HE (1 mg/ml) was prepared by adding 125 µl of stock solution to 9.875 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (DMEM/FCS).

#### Cell Aggregation Assay

Aggregation between melanoma cells was measured by a double color assay. Cells were harvested by short trypsinization of subconfluent monolayers, washed three times with DMEM/FCS, and resuspended at a final concentration of  $2 \times 10^6$  cells/ml. Two separate suspensions of cells ( $2 \times 10^6$ /ml) were stained fluorescent green or fluorescent red by incubation for 60 minutes at 37°C with SFDA (50 µg/ml in DMEM/FCS) or with HE (40 µg/ml in DMEM/FCS), respectively. Thereafter, cells were washed three times with DMEM/FCS and resuspended in DMEM/FCS at a final concentration of  $1 \times 10^6$  cells/ml. Fluorescent green cells (50 µl) were mixed with fluorescent red cells (50 µl) and incubated for 10 and 30 minutes at 37°C. After incubation, cells were fixed by adding 50 µl of ice-cold paraformaldehyde (1.5% w/v in DMEM/FCS). The samples were kept on ice in the dark for 30 minutes and subsequently analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). A total of 10,000 fluorescent events were counted. The results were expressed as the percentage of double colored events of the total of green plus red events.

### Eukaryotic *memD* Expression Vectors

A 5' 0.34-kb *memD* fragment was amplified by using the polymerase chain reaction primers 5'-atggaatc-caagggggc-3' (sense, including the translation start codon) and 5'-tagcatgcacacaaatc-3' (antisense, posi-

tions 326–342). This polymerase chain reaction fragment was cloned into the pGEM-T vector (Promega, Madison, WI), isolated via *SphI/SacII* digestion, and cloned into the *SphI/SacII* digested *memD* cDNA clone pJG456. This complete *memD* cDNA clone (pEG25) was extensively sequenced to ensure that no mutations were introduced.

pEG25 was used to make the eukaryotic *memD* expression vectors pWD200 and pWD201. pWD201 is the blunt-ended 5' 2.1-kb *SacI/HindIII* (including the translation start codon) fragment of pEG25 ligated into the blunt-ended *BamHI* site of the neomycin resistant pZIPneo vector<sup>28</sup> in the sense orientation. pWD200 is the antisense variant of pWD201.

### Electroporation

Plasmid DNA was purified by using a Qiagen Plasmid purification kit (Qiagen, Chatsworth, CA). Cells were washed two times with PBS, and  $2 \times 10^7$  cells were mixed with 20  $\mu$ g of DNA construct carrying the G418 resistance gene in 500  $\mu$ l of PBS. After an incubation of 10 minutes on ice, cells were electroporated in a 1-ml electroporation cuvette (Eurogentec, Belgium) by a 2000-volt pulse from an ISCO type 494 power supply so that cell survival was 60 to 70%. Cells were incubated on ice for 10 minutes and replated on 10-cm Petri dishes (Nunc, Naperville, IL). Stable transfectants were selected in the presence of 1 mg/ml G418 in the culture medium. After selection, cells were grown in the presence of 0.5 mg/ml G418.

## Results

### Cloning and Characterization of *memD*

From a differential mRNA display comparing a non- and a highly metastasizing human melanoma cell line, nine cDNA clones were isolated, which were differentially expressed in a panel of human melanoma cell lines with different metastasizing potential.<sup>10</sup> On a Northern blot with total RNA from this panel of cell lines, one clone (clone 3) showed expression of a 4.6-kb transcript in the highly metastasizing human melanoma cell lines BLM and MV3, whereas expression was very weak or not detectable in the other melanoma cell lines (Figure 1A). In the following studies we only included the 530, 1F6, BLM, and MV3 melanoma cell lines. Overexposure of a Northern blot resulted in a weak signal in the nonmetastasizing human melanoma cell line 1F6, whereas no detectable signal was present in 530 cells (Figure 1B). The observed differences in expression of clone 3 in our panel of human melanoma cell lines cannot be ascribed to major chromosomal rearrangements or gene amplifications as was demonstrated by Southern blot analysis (Figure 2).

Screening of an MV3 derived cDNA library (in the vector  $\lambda$ Zap)<sup>10</sup> resulted in the isolation of cDNA clone pJG456. This clone was designated *memD* (melanoma metastasis clone D). A computer database search revealed that the *memD* cDNA clone was identical (99.9% identity in a 2478-bp overlap) with the human activated

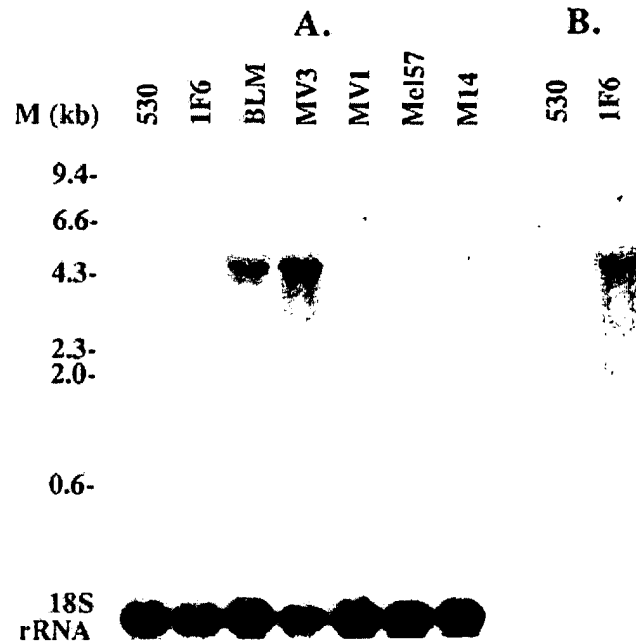


Figure 1. *memD* expression in a panel of human melanoma cell lines. A: Northern blot analysis of *memD* mRNA expression in a panel of human melanoma cell lines. B: Overexposure of a Northern blot showing a weak *memD* expression in the 1F6 human melanoma cell line.

leukocyte cell adhesion molecule (ALCAM).<sup>11</sup> *memD* also showed similarity (75% identity on the protein level) with the chicken neural adhesion molecule BEN/SC1/DM-GRASP<sup>12–14</sup> and with the melanoma cell adhesion molecule Mel-CAM/MUC18 (26% identity and 47% similarity on the protein level).<sup>15</sup>

Sequence comparison between the 4235-nucleotides long *memD* cDNA clone (Y10183 in EMBL/GenBank) and the ALCAM cDNA clone (L38608 in EMBL/GenBank) revealed that *memD* is extended at the 3'-end with 1721 nucleotides (positions 2472–4193, poly(A) tail excluded) and lacks 66 nucleotides at the 5'-end including the translation start codon. There are also two differences at the nucleotide level in the region of overlap. First, the C at nucleotide position 899 (C899) in *memD* (T965 in the ALCAM cDNA clone) results in the substitution of amino acid M301 (in ALCAM) to T301 (in MEMD). Second, nucleotide G1862 (in ALCAM) is absent in the 3' untranslated region of the *memD* cDNA clone. The significance of these differences is not known.

### MEMD/ALCAM Expression

J3–119,<sup>24</sup> a monoclonal anti-ALCAM antibody that recognizes the second amino-terminal V-type domain (V2) of the ALCAM protein,<sup>29</sup> was used to study the distribution pattern of the MEMD/ALCAM protein in the human melanoma cell lines 530, 1F6, BLM, and MV3. Expression of MEMD/ALCAM was mainly concentrated in areas involved in cell-cell interactions between clustered BLM cells and clustered MV3 cells (Figure 3). Expression in 1F6 was sporadically observed in some clustered cells corresponding with the weak *memD* mRNA expression in

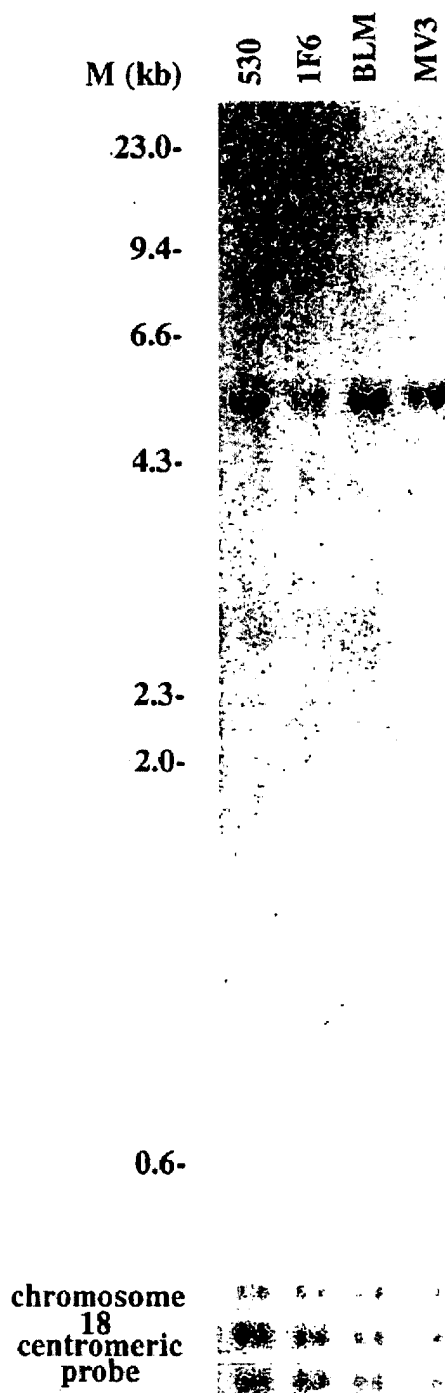


Figure 2. Southern blot analysis of memD in a panel of human melanoma cell lines.

this cell line. MEMD/ALCAM expression was not detected in the 530 cell line (not shown).

### Aggregation of Human Melanoma Cells

Adhesion molecule homologs of the MEMD protein, ie, BEN/SC1/DM-GRASP and ALCAM, were reported to mediate homophilic and heterophilic adhesion interactions.<sup>11,13,17,30</sup> This and the observation that MEMD/ALCAM expression is concentrated in areas of contact

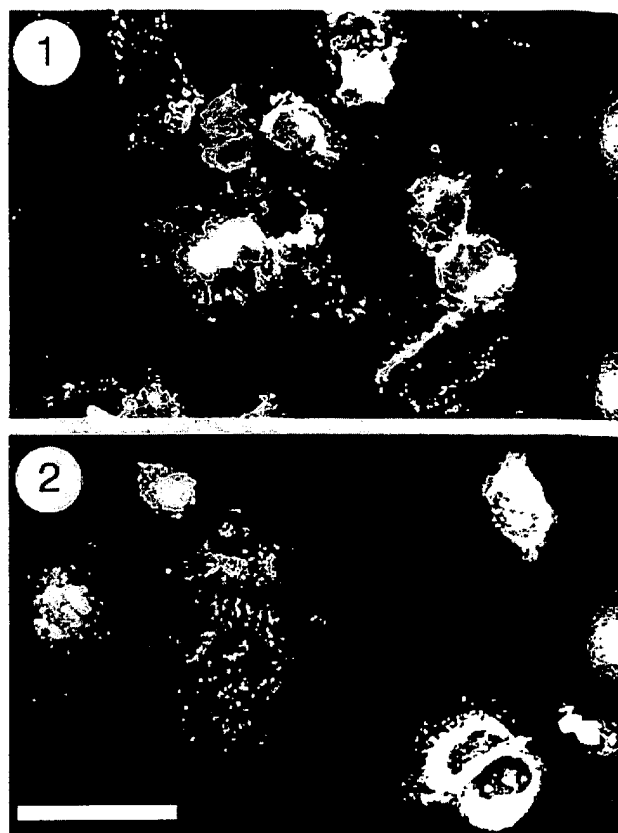


Figure 3. Immunolocalization of MEMD/ALCAM in cultured BLM (1) and MV3 (2) human melanoma cells. A representative staining is shown. bar, 50  $\mu$ m.

between cells, as was reported for the surface glycoprotein BEN,<sup>12</sup> raised the question whether MEMD/ALCAM was involved in mediating melanoma cell-cell adhesion. For this purpose, the human melanoma cell lines 530, 1F6, BLM, and MV3 were tested in an aggregation assay. A prior FACS analysis showed that all melanoma cell lines were CD6 negative (data not shown), excluding the possibility of MEMD/ALCAM-CD6 adhesive interactions.

Fluorescent red- and green-stained cells were analyzed in a FACScan flow cytometer for the capacity to form mixed cell clusters. The MEMD/ALCAM positive human melanoma cell lines BLM, MV3, and 1F6 indeed aggregate as shown in Figure 4A. Aggregation between the MEMD/ALCAM-positive BLM cell line and the other melanoma cell lines is shown in Figure 4B. MEMD/ALCAM-positive cell lines aggregate with BLM, ie, 1F6 and MV3. In all cases, 530 hardly aggregates.

We tested whether the available anti-ALCAM antibodies J3-119 and J4-81,<sup>24</sup> which both recognize the second amino-terminal V-type domain (V2) of the ALCAM protein,<sup>29</sup> could be used to inhibit cell adhesion to provide a more direct proof of the role of the MEMD/ALCAM protein. However, the antibodies did not inhibit the adhesive interactions, probably because the homophilic cell-cell adhesive interactions are mediated via the C-type domains.<sup>29,31</sup> The only monoclonal antibody known to block MUC18 mediated cell-cell interactions recognizes the glycosylated HNK-1 epitope.<sup>5</sup> We established that this epitope is not present on MEMD/ALCAM expressed

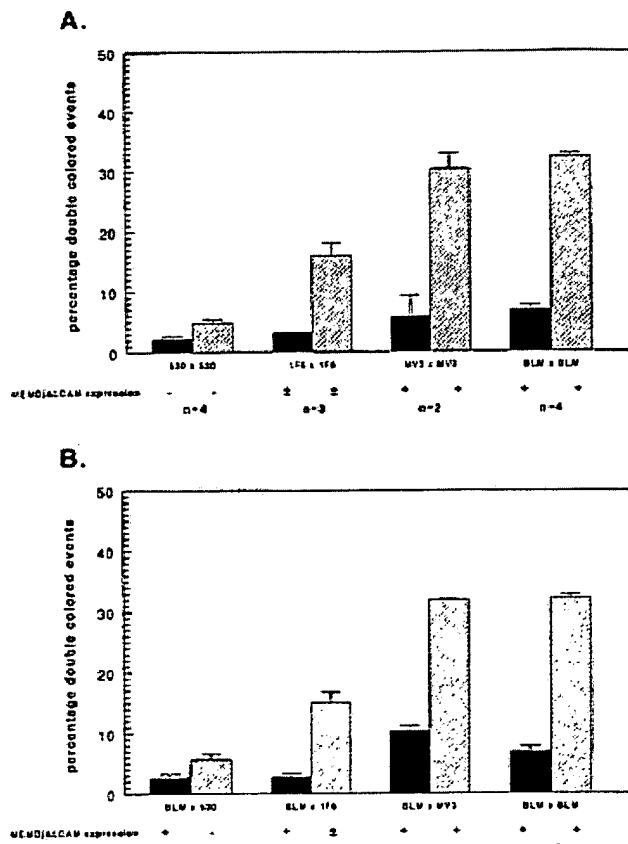


Figure 4. Aggregation of cultured human melanoma cells. A: Aggregation between identical cells. B: Aggregation between BLM cells and 530/1F6/MV3 cells. Data are presented as mean  $\pm$  SD of *n* different experiments. Black bars, aggregation after 10 minutes; shaded bars, aggregation after 30 minutes. MEMD/ALCAM expression in the cultured human melanoma cell lines is indicated as high (+), weak ( $\pm$ ), or absent (-).

by the melanoma cell lines (not shown). It is known that glycosylation of BEN also does not effect its homophilic binding properties.<sup>17</sup>

### Aggregation of 530 Cells Transfected with *memD*

To provide a more direct proof of the role of the MEMD/ALCAM protein in homophilic cell-cell aggregation in our panel of human melanoma cell lines, 530 cells (lacking *memD*/MEMD expression) were transfected with the sense (pWD201) and antisense (pWD200) *memD*-pZIPneo expression constructs and pZIPneo alone. In a selection of transfected G418-resistant cell clones (10 sense, seven antisense, and four empty vector controls), all transfectants displayed the expected *memD* mRNA expression profile (not shown). From this collection of transfectants, two sense (201-3 and 201-11) and one antisense (200-7) *memD* transfectant, and one control (ZIP-1) were analyzed in more detail by immunofluorescence using the monoclonal anti-ALCAM antibody J3-119.<sup>24</sup> Expression of the MEMD/ALCAM protein in the sense 530-*memD* transfectants 201-3 and 201-11 is mainly concentrated in areas involved in cell-cell interactions between clustered transfected 530 cells (Figure 5,

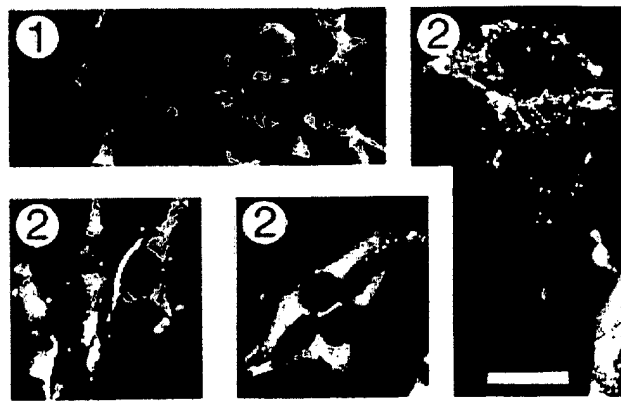


Figure 5. Immunolocalization of MEMD/ALCAM in 530 cells transfected with *memD*. 1: A representative staining is shown of negative controls (parental 530 cells, 530 cells transfected with pZIPneo (ZIP-1), or antisense *memD* (200-7)). 2: Representative stainings of 530 cells transfected with sense *memD* (201-3 or 201-11). Scale bar, 25  $\mu$ m.

part 2) as we also found for BLM and MV3 cells (Figure 3). The controls (parental 530, antisense *memD* 200-7, and ZIP-1) are negative (Figure 5, part 1).

To further test whether MEMD/ALCAM is functionally involved in melanoma cell-cell interactions, we performed a FACS analysis on the 530-*memD* transfectants. The results of this analysis showed that the sense 530-*memD* transfectants 201-3 and 201-11 do aggregate, whereas the antisense transfectant 200-7 and the control ZIP-1 do not (Figure 6A). An aggregation analysis between the MEMD/ALCAM-positive BLM cell line and the 530-*memD* transfectants gave a similar result. BLM cells only aggregate with the sense 530-*memD* transfectants 201-3 and 201-11 (Figure 6B). These assays indicate that the expression of *memD*/MEMD is apparently sufficient and essential for cell-cell interactions in the human melanoma cell lines tested.

### Expression of *memD* in Human Organs, Cutaneous Melanocytic Lesions, and Tumor Cell Lines

To gain some insight in its distribution, a Northern blot containing total RNA from several human organs was screened for *memD* mRNA expression (Figure 7). *memD* is expressed in spleen, placenta, and liver. A weak expression is visible in lung. For an additional characterization, a Northern blot containing total RNA from several tumor cell lines was screened for *memD* mRNA expression. Within this panel of cell lines, a strong expression could be detected in the untransformed Ayca-1 human foreskin fibroblasts, A-431 epidermoid carcinoma, T24 bladder carcinoma, and HT-1080 fibrosarcoma cell lines. This expression level was comparable with *memD* expression in the BLM and MV3 cell lines after the same time of exposure (not shown here). A weaker expression was detected in the PC-3 prostate adenocarcinoma, MCF-7 mammary carcinoma, and 143B PML BK TK osteosarcoma cell lines (Figure 8). These results indicate a restricted distribution of *memD* in human tumor cell lines, although it is detectable in several healthy human organs.

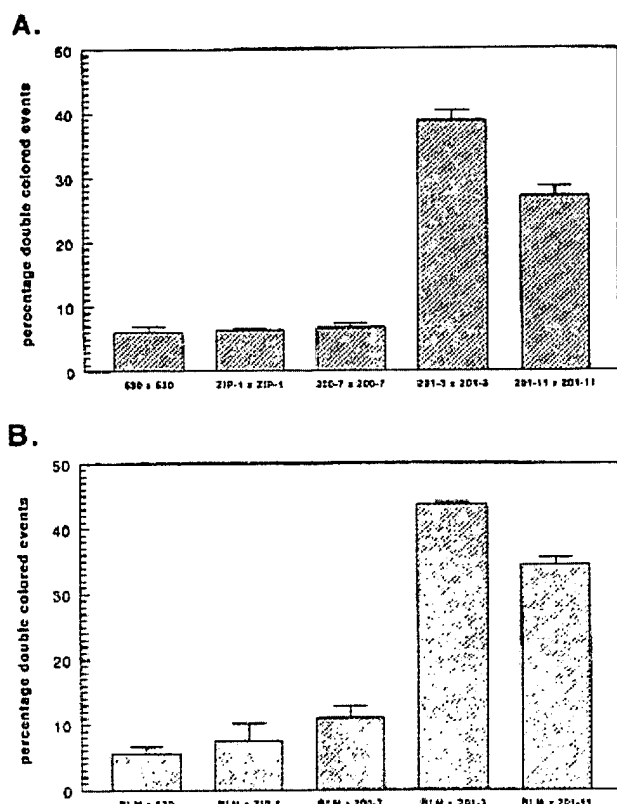


Figure 6. Aggregation of 530 cells transfected with *memD*. A: Aggregation between 530-*memD* cells. B: Aggregation between BLM cells and 530-*memD* cells. Data are presented as mean  $\pm$  SD of  $n = 3$  different experiments. ZIP-1, 530-pZIPneo transfectant; ZIP-7, antisense 530-*memD* transfectant; 201-3 and 201-11, sense 530-*memD* transfectants.

The expression of *memD* mRNA in several human cutaneous melanocytic lesions was examined by Northern blot analysis of total RNA isolated from several human melanoma metastasis lesions (Figure 9). *memD* is strongly expressed in cultured pericytes and moderate in melanocytes and cultured endothelial cells. A weak expression is detectable in xenografts derived from the radial and vertical growth phase and in 2 of 13 melanoma metastasis lesions (MM#1 and MM#4). Expression in MM#5 and MM#6 is moderate and comparable with *memD* expression in endothelial cells. Whereas it is known that the expression of progression marker genes

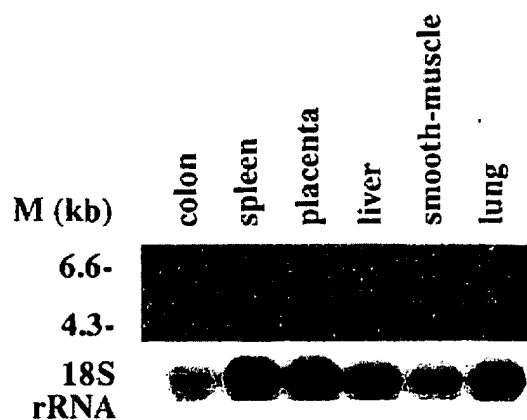


Figure 7. Northern blot analysis of *memD* mRNA expression in several human organs.

of melanoma is induced under conditions required for culturing melanocytes, expression of *memD*/ALCAM in pericytes is in agreement with the findings of Patel et al.<sup>32</sup> Expression in endothelial cells was not reported before.

### Discussion

In this study we describe *memD*, a gene differentially expressed in a panel of human melanoma cell lines with different metastasizing potential. *memD* is identical to ALCAM and is a new adhesion molecule not earlier found in human cutaneous melanoma. Expression of *memD*/MEMD was found primarily in highly metastasizing human melanoma cell lines and correlated with the capacity of cells to form aggregates (Figure 4). We have provided evidence that the expression of *memD*/MEMD is sufficient and essential for cell-cell interactions of the human melanoma cell lines analyzed, as ectopic *memD*/MEMD expression in the *memD*/MEMD negative cell line 530 induced these cells to aggregate with themselves and with BLM cells (Figure 6). The involvement of *memD*/MEMD in adhesive cell-cell interactions is also supported by its prominent presence in areas of contact between melanoma cells (Figures 3 and 5).

ALCAM was recently identified as a CD6 ligand and is expressed on activated leukocytes, fibroblasts, epithelial,

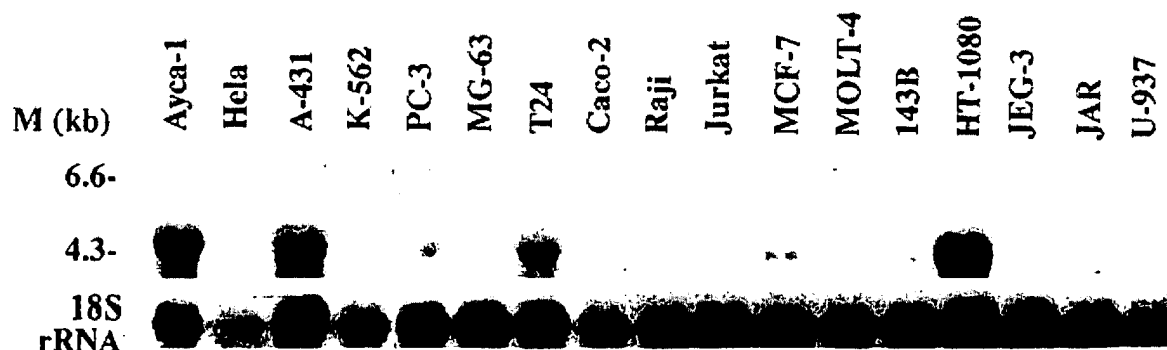


Figure 8. Northern blot analysis of *memD* mRNA expression in a panel of human tumor cell lines. The cell lines used were: Aycal-1 human fibroblasts, Hela cervix carcinoma, A-431 epidermoid carcinoma, K-562 chronic myelogenous leukemia, PC-3 prostate adenocarcinoma, MG-63 osteosarcoma, T24 bladder carcinoma, Caco-2 colon carcinoma, Raji Burkitt lymphoma, Jurkat lymphoma, MCF-7 mammary carcinoma, MOLT-4 acute lymphoblastic leukemia, 143B PML BK TK osteosarcoma, HT-1080 fibrosarcoma, JEG-3 choriocarcinoma, JAR placenta choriocarcinoma, and U-937 histiocytic lymphoma.

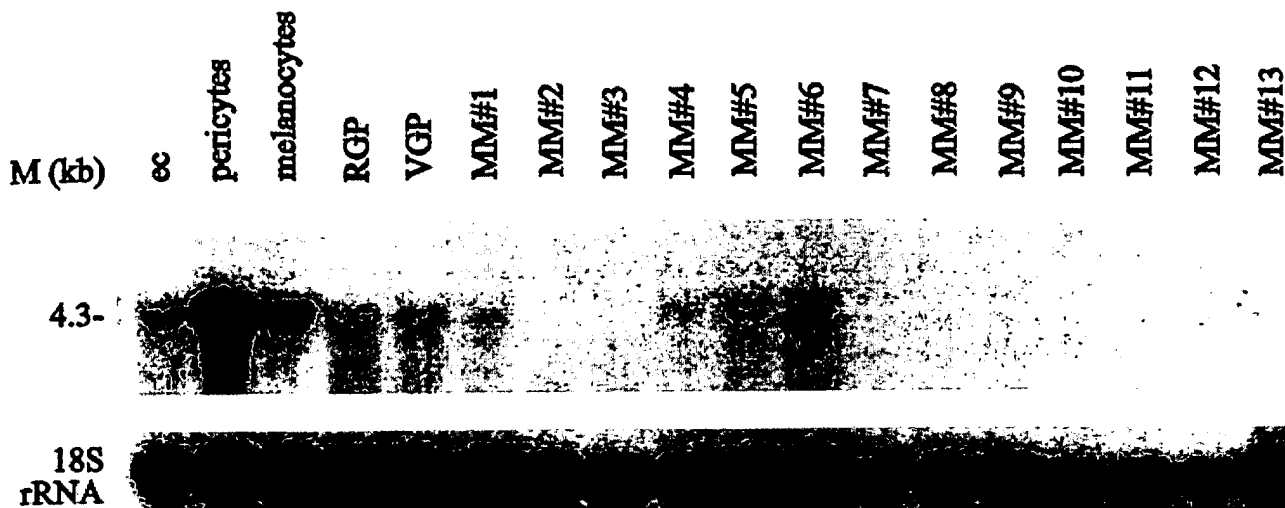


Figure 9. Northern blot analysis of *memD* mRNA expression in human cutaneous melanocytic lesions. ec, endothelial cells; RGP, xenograft derived from radial growth phase; VGP, xenograft derived from vertical growth phase; MM, melanoma metastasis lesions.

and neural cells.<sup>11,32</sup> Homophylic and heterophylic ALCAM adhesion interactions have been reported. Heterophylic ALCAM-CD6 adhesion interactions mediate binding of activated leukocytes to T cells and of thymic epithelial cells to thymocytes. Yet little is known about homophylic ALCAM-ALCAM cell adhesion interactions.<sup>11</sup> In the melanoma cell lines we used, heterophylic MEMD/ALCAM-CD6 interactions could be excluded because all cell lines were CD6 negative. Furthermore, the adhesion molecules MUC18 and ICAM-1 are not involved in this cell-cell adhesion because the *memD*/MEMD positive melanoma cell lines do not express MUC18<sup>20</sup> or ICAM-1 (Y. van Kooyk, unpublished results). Thus, we assume that our investigations concern the involvement of MEMD/ALCAM expression in homophylic melanoma cell interactions. Its chicken homolog BEN (for bursal epithelium and neurons), a neural adhesion molecule, also behaves as a homophylic cell adhesion molecule.<sup>17</sup> BEN protein expression was concentrated on the surface of neuron cells in areas involved in cell-cell interactions.<sup>12</sup> Similarly, we showed that the MEMD/ALCAM protein expression is mainly concentrated in areas of contact between parental cells and between cells transfected with *memD* (Figures 3 and 5). The similar cellular distribution and the homology with BEN indicated that MEMD/ALCAM is involved in homophylic cell interactions and probably contains an adhesion activity.

The results of an aggregation assay performed on our panel of human melanoma cell lines are in agreement with these observations. The assays revealed a clear correlation between *memD*/MEMD expression and cell aggregation (Figure 4). BLM and MV3, cell lines with a high *memD*/MEMD expression, displayed a higher aggregation percentage than 1F6, a cell line with a weak expression, whereas 530 cells (lacking *memD*/MEMD expression) do not aggregate. By comparing the percentages of 1F6-1F6 and BLM-1F6 aggregation, it was remarkable that they were almost the same. This suggests that the low levels of MEMD/ALCAM expression in 1F6 enable cells to aggregate to a limited extent. Importantly,

as parental 530 cells lack *memD*/MEMD expression and cannot form cell clusters at all, after transfection of *memD*/MEMD in these cells they show an aggregational behavior that is comparable with the BLM cells (Figure 6A). Moreover, the *memD*/MEMD transfected 530 cells also aggregate with parental BLM cells (Figure 6B). Together, these findings strongly support our conclusions that MEMD/ALCAM is a cell adhesion molecule necessary for homophylic cell-cell aggregation. The observation that parental 530 cells cannot aggregate with BLM cells whereas the transfected 530-*memD* cells can strongly indicates that expression of MEMD/ALCAM is sufficient and essential for cells to aggregate. Nevertheless, our data do not formally exclude the involvement of other co-acting factors in the aggregational behavior of the human melanoma cells tested.

The results presented in this study identify MEMD/ALCAM to be a new adhesion molecule for human cutaneous melanoma, the expression of which in a panel of human melanoma cell lines is correlated with cell aggregation and metastatic potential. As MEMD/ALCAM is involved in both homophylic and heterophylic cell-cell adhesion interactions, it may be important during neoplastic progression. Both homophylic and heterophylic interactions between tumor cells and cells of normal tissues (eg, fibroblasts, epithelial cells, expressing functional MEMD/ALCAM, and/or CD6)<sup>11,32</sup> could be involved in the dynamic interactions of mobile tumor cells with their environment necessary for successful metastasis. In this light, it may seem disappointing that *memD* expression is detected in only 4 of 13 melanoma metastasis lesions tested (Figure 9). However, it is clear that local tumor growth, either in a primary or metastasis lesion, requires other properties than cell motility through tissue barriers. Activated leukocytes (T cells, B cells, and monocytes) and highly metastasizing melanoma cells must have migrational activity in contrast to locally growing tumor cells. We postulate that certain properties related with high mobility of cells (including constitutive MEMD/ALCAM expression) might be less compatible with local out-

growth, and their expression should preferably be transient. The preliminary results of *memD* expression in melanoma metastasis lesions should be confirmed by immunohistochemical studies. Moreover, binding of metastasizing cells (single cells or cell clusters) to T cells via MEMD/ALCAM-CD6 or MEMD/ALCAM-MEMD/ALCAM interactions could also be a mechanism to help these cells to circumvent the immune surveillance and thereby enhance metastasis. In that case, suppression of *memD*/MEMD expression and/or function should be a tool to inhibit tumor cells to metastasize. A clue for the involvement of MEMD/ALCAM during neoplastic progression may be provided by the homology with MUC18,<sup>15</sup> another member of the immunoglobulin superfamily. MUC18 is a cell surface adhesion molecule that is strongly expressed by advanced primary and metastatic melanoma lesions.<sup>4</sup> Transfection of MUC18 in nonmetastatic MUC18-negative primary cutaneous melanoma cells increased their metastatic potential in nude mice.<sup>33</sup> Additional studies are therefore needed to clarify the role of *memD*/MEMD in tumor invasion and progression.

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